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Physiology

Molecular basis for decreased muscle chloride conductance in the myotonic goat

(chloride channel/myotonia/action potential/electrophysiology/skeletal muscle)

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ABSTRACT **Certain forms of myotonia, a condition characterized by delayed relaxation of muscle secondary to sarcolemmal hyperexcitability, are caused by diminished chloride conductance in the muscle cell membrane. We have investigated the molecular basis for decreased muscle chloride conductance in the myotonic goat, an historically important animal model for the elucidation of the role of chloride in muscle excitation. A single nucleotide change causing the substitution of proline for a conserved alanine residue in the carboxyl terminus of the goat muscle chloride channel (gClC-1) was discovered. Heterologous expression of the mutation demonstrated a substantial (+47 mV) shift in the midpoint of steady-state activation of the channel, resulting in a diminished channel open probability at voltages near the resting membrane potential of skeletal muscle. These results provide a molecular basis for the decreased chloride conductance in myotonic muscle.**

Electrical excitation of the skeletal muscle cell membrane critically depends upon its ability to undergo rapid changes in selective ionic permeabilities that serve in the generation and propagation of action potentials. In a condition known as myotonia, abnormalities in specific sarcolemmal ion conductances can lead to a reduced electrical threshold for firing action potentials and in conduction of repetitive impulses that result in sustained muscle fiber contraction (1). Myotonia is characterized clinically by delayed relaxation of muscle after sudden forceful contractions and is associated with a variety of acquired and hereditary diseases (2). Congenital myotonia without associated weakness or muscular dystrophy can be transmitted as either an autosomal dominant (myotonia congenita or Thomsen disease) or recessive (recessive generalized myotonia or Becker myotonia) trait.

The current hypotheses regarding the pathophysiology of myotonia congenita were initially formulated from studies of the myotonic goat, an unusual breed afflicted with severe autosomal dominant congenital myotonia that closely resembles human Thomsen disease (3, 4). These animals are often referred to as "fainting," "nervous," "stiff-legged," or "epileptic" goats because of their tendency to develop severe acute muscle stiffness and become immobile (and often fall) when attempting to make sudden forceful movements or when startled.

The pathogenesis of myotonia in the goat was elucidated by Bryant and colleagues (5, 6) who first described a severely diminished resting chloride conductance (g_{Cl}) in muscle fibers from affected animals. This group (7) also demonstrated that myotonia could be produced in normal skeletal muscle fibers bathed in a Cl^--free solution. These important observations and subsequent studies contributed to the understanding of the role of g_{Cl} in normal sarcolemmal excitation and in the pathophysiology of myotonia. Many years later, molecular genetic studies on murine (8, 9) and human (10–13) myotonia confirmed structural and functional defects in a skeletal muscle chloride channel (termed ClC-1).

In view of the pivotal role played by the myotonic goat in establishing the pathophysiology of myotonia, we sought to define the molecular basis for diminished g_{Cl} in the myotonic goat by directly examining the ClC-1 muscle chloride channel for mutations. We report herein the discovery of a single nucleotide change in goat ClC-1 resulting in a missense mutation that causes a significant functional disturbance in the channel that fully explains decreased sarcolemmal g_{Cl} and the myotonic phenotype.

MATERIALS AND METHODS

Isolation of RNA. Skeletal muscle and other tissues were obtained from a phenotypically and electromyographically normal goat (wild type, WT) and from a myotonic goat (generously provided by V. LeQuire, Franklin, TN) taken from a colony that has been inbred for several generations. The colony consists of 13 goats from two successive generations, of which all are affected with severe myotonia. The affected animal used in the study exhibited prominent percussion and action myotonia, generalized muscular hypertrophy, and frequent bursts of repetitive discharges (myotonic runs) on an electromyograph. Protocols were approved by the Animal Care Committee of Vanderbilt University. Isolation of total and poly(A)$^+$ RNA was performed with standard methods (14, 15).

Northern Blot Analysis. Total RNA (10 μg) was size-fractionated on denaturing 1% agarose/6% (vol/vol) formaldehyde gels and transferred to a nylon membrane (Hybond-N, Amersham). The blot was probed with a [^{32}P]dCTP-labeled antisense RNA probe transcribed from human ClC-1 (hClC-1) cDNA (nt 1–1415) using T7 RNA polymerase. Hybridization was performed at 50°C for 16 h in 50% formamide/5× SSPE (1× SSPE is 0.18 M NaCl/10 mM Na_2HPO_4/1 mM EDTA)/1% SDS/0.2% Ficoll/0.2% polyvinylpyrrolidone/2 mM sodium pyrophosphate/0.05 M Tris·HCl, pH 7.5/25 mM EDTA/1% BSA/^{32}P-labeled RNA probe at 3×10^6 cpm/ml, followed by washes in 0.1× standard saline citrate (SSC)/0.1% SDS at 60°C.

Isolation and Cloning of Goat ClC-1 cDNA. A directional cDNA library was constructed from myotonic goat skeletal muscle in the plasmid pSPORT1 using 5 μg of poly(A)$^+$ RNA and the SuperScript cDNA synthesis system (Life Technologies, Gaithersberg, MD). Approximately 250,000 unamplified recombinants were screened by standard methods (14). Duplicate colony lifts were hybridized at 42°C for 16 h in 50%

Abbreviations: g_{Cl}, chloride conductance; WT, wild type; RACE, rapid amplification of cDNA ends; h, human; g, goat.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U60275).

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formamide/5× SSPE/4× Denhardt's solution/1% SDS/ denatured salmon sperm DNA at 0.3 mg/ml with [^{32}P]dCTP-labeled hClC-1 cDNA (*Not*I–*Bsp*H1 fragment, nt 1–1415) at 0.8 × 10^6 cpm/ml. Membranes were washed twice in 5× SSC/0.1% SDS at 60°C. Positive clones were colony-purified.

Extensions of the cDNA to the 5′ end were made using either reverse transcriptase-coupled PCR or the 5′ rapid amplification of cDNA ends (RACE) method (16) with modifications (17). First-strand cDNA synthesis was performed with either random primers or a nondegenerate gene-specific primer (920RT, 5′-CAAAGGCGCTGAACGTGGCTGCA-A-3′). In reverse transcriptase-coupled PCR experiments, a degenerate forward primer based on the amino terminus of hClC-1 [56F, 5′-(C/A)CCCNCA(A/G)TA(T/C)CA(A/G)T-A(T/C)ATG-3′] was paired with a reverse nondegenerate goat ClC-1 (gClC-1) primer (897R, 5′-ATCCTCGCCAGTAGTT-CCTC-3′). In 5′-RACE experiments, PCR amplifications were done with a $(dT)_{17}$-adaptor, a gene-specific primer (72R, 5′-AGTCCCAGGCGGTGCATACA-3′, or 174R, 5′-GTTT-TGGCACTGACATAATC-3′), and a modified adaptor primer (5′-GACTCGAGTCGACATCGTT-3′). In each 5′ cloning experiment, specific products were identified by Southern blot hybridization using a ^{32}P-labeled hClC-1 cDNA probe, subcloned into plasmids, and sequenced by the Sanger dideoxynucleotide-mediated chain-termination method.

Single-Strand Conformational Analysis. First-strand synthesis was carried out with randomly primed total RNA (4 μg) from either normal or myotonic goat skeletal muscle. After first-strand cDNA synthesis, double-stranded cDNAs were amplified by two sequential PCRs. Initially, three overlapping regions (0.9–1 kb) of gClC-1 were amplified using three sets of primers. Each fragment was purified by spin column chromatography (Qiagen, Chatsworth, CA) and used as the template for the secondary PCRs containing nested primer pairs to produce 300- to 400-bp products. Each secondary PCR mixture (10 μl) contained 2 μl of the first-round PCR product, each primer at 0.5 μM, all four dNTPs (each at 70 μM), 1× PCR buffer (10 mM Tris·HCl, pH 8.9/50 mM KCl), 1.5–3.5 mM $MgCl_2$ (optimized for each primer set), 0.1 μl of [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq), and 0.25 unit of *Taq* polymerase (5 units/μl). Cycling conditions were initial denaturation of 8 min at 94°C, followed by 30 cycles of 94°C for 1 min, annealing temperature for 1 min (optimized for each primer set), and 72°C for 1 min. Products were denatured and then electrophoresed on nondenaturing 0.5 MDE gels (J.T. Baker) at 8 W for 16 h at 25°C. Abnormal conformers were excised from the dried gels, reamplified, and directly sequenced using fluorescein dye-labeled terminator chemistry on an Applied Biosystems model 373 automated sequencer. Genotyping was performed by *Mbo*II digestion of a 177-bp fragment PCR-amplified from genomic DNA (100 ng) using primers 2560F (5′-AGGAGCTGCAGAAGGCCATT-3′) and 2717R (5′-TCATGTCCCCTGCCCCAGTG-3′) with 35 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min.

Mutagenesis. Site-directed mutagenesis of hClC-1 was performed by overlap-extension PCR mutagenesis (18) using the following oligonucleotides: 2298F (5′-CATCTTCCAGTCCC-TGCTTC-3′), 2674R (5′-AAGTCGTGTTCCGGAAGCTG-GGAAGGGGAGGGCGGAGCT-3′), 2636F (5′-AGCTCC-GCCCTCCCCTTCCCAGCTTCCGGAACACGACTT-3′), and 2829R (5′-GAGGCGAATTCTAGACCCTATACCTTG-CCTGGG-3′). Final PCR products were digested with *Sac*I and *Bsu*36I, and a 392-bp fragment containing the mutation A885P was directionally ligated into the pSP64T-hClC-1 construct (13). Presence of the mutation and absence of polymerase errors in the *Sac*I–*Bsu*36I region in three independent recombinants were confirmed by dideoxynucleotide sequencing.

Functional Expression in *Xenopus* Oocytes. Purified plasmid constructs of A885P-hClC-1 and WT-hClC-1 were transcribed *in vitro* and expressed in *Xenopus laevis* oocytes as described (13). Standard two-electrode voltage clamp recordings were made and analyzed as described (13) except that a Warner OC-725C amplifier (Warner Instrument, Hamden, CT) was used. Data are shown as mean ± SEM.

Current–voltage relationships were assessed from voltage steps between −145 mV and +35 mV by plotting either the current amplitude measured directly after settling of the capacitive transient (instantaneous current–voltage relationship) or at the end of a 660-ms test pulse (steady-state current–voltage relationship). To construct activation curves, instantaneous current amplitudes measured after 1.4-s prepulses to different voltages were normalized by dividing by the maximal value recorded at a fixed potential of −135 mV. These values were plotted against the preceding potential as described (13). The plot shows the voltage dependence of the relative open probability (P_{open}) at the end of the prepulse. Steady-state activation curves obtained in this manner were fitted with a single Boltzmann term and a voltage-independent value: $I(V) = \text{Amp} \cdot \{1 + \exp[(V - V_{0.5})/k_V]\}^{-1} + \text{constant}$, where Amp is an amplitude term, k_V is a slope factor, and $V_{0.5}$ approximates the voltage at which 50% of channels are activated.

RESULTS

To verify the presence of an mRNA transcript encoding a skeletal muscle chloride channel and to exclude large-scale mutations (insertions or deletions) in this gene, we performed Northern blot analysis using a radiolabeled probe derived from hClC-1 (nt 1–1415) representing sequence conserved among ClC channels (Fig. 1). Under reduced stringency conditions, the hClC-1 probe hybridized to an ≈3.6-kb transcript of near equal intensity in both normal and myotonic goat skeletal muscle total RNA but did not hybridize to RNA from goat brain, heart, liver, or kidney. A second weaker hybridization signal was also observed at ≈4.5 kb in both skeletal muscle lanes with near equal intensity. The latter signal is believed to result from either cross-hybridization to another mRNA species, presence of incompletely processed ClC-1 mRNA, or an undefined ClC-1 splice variant. These data indicate the presence of a ClC-1 homologue that is selectively expressed in muscle. The similarity of transcript sizes in normal and myotonic goat rules out large deletion or insertion mutations in this gene.

Having ruled out gross abnormalities in the size or expression levels of the mRNA, we searched for mutations at the level of nucleotide sequence. We constructed a cDNA library from myotonic goat skeletal muscle and cloned the myotonic goat skeletal muscle chloride channel cDNA. The longest of five positive cDNAs obtained from library screening (clone B8, 2.4 kb) was sequenced, and an open reading frame of 2107 nt

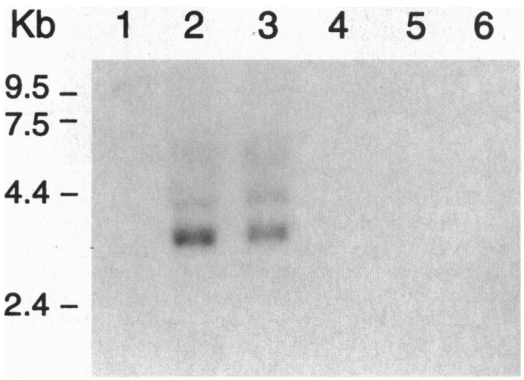

FIG. 1. Northern blot analysis of ClC-1 in goat tissues. The blot was hybridized with a human ClC-1 probe. Each lane contains 10 μg of total RNA isolated from the following tissues: brain (lane 1), normal skeletal muscle (lane 2), myotonic muscle (lane 3), heart (lane 4), kidney (lane 5), and liver (lane 6).

that terminated in a stop codon was identified. The other four positive clones were examined by restriction digest analysis, partially sequenced, and determined to be similar to B8. The nucleotide sequence of the open reading frame of B8 exhibits 87% identity with hClC-1 and 83% with rat ClC-1 but shares less identity with other ClC isoforms (61% with ClC-2, 53% with ClC-0, and 25% with ClC-3). The location of the stop codon corresponds to the stop codon present in hClC-1 (nt 2965). This was consistent with the sequence representing a partial-length goat ClC-1 cDNA (designated gClC-1). We used additional PCR techniques (reverse transcriptase-coupled PCR and 5′-RACE) to extend the sequence in the 5′ direction. The 5′ end was extended an additional 829 nt but did not reach a start codon. The assembled sequence of gClC-1 was found to encode a protein with 87% amino acid identity with hClC-1, 83% with rat ClC-1, and 48% with rat ClC-2. Inspection of the sequence aligned with hClC-1 revealed no obvious insertion, deletion, or nonsense mutations.

The absence of obvious discernable mutations at this stage in the analysis of gClC-1 necessitated a systematic approach to identify single nucleotide differences. To screen gClC-1 for single nucleotide mutations, we used single-strand conformational analysis (19) adapted for use with RNA as the starting material. Approximately 54% of the cDNA sequence of gClC-1 was screened by this method. An abnormal conformer was found in a segment encoding a portion of the carboxyl terminus of the channel (Fig. 2*A*). Conformers from both the WT and myotonic samples were eluted from the gel, re-amplified using PCR, and directly sequenced on both strands. Two sequence differences between WT and myotonic samples were identified. One difference was a T → C transition in the myotonic sample that does not result in an amino acid change and is most likely a polymorphism. We also detected a G → C transversion in the myotonic sample (Fig. 2*B*) that results in an alanine (*G*CC) → proline (*C*CC) substitution predicted to occur in the carboxyl terminus of gClC-1 104 amino acids from the termination codon (corresponding to amino acid 885 in hClC-1). This transversion creates a new *Mbo*II restriction site thus providing the basis for an allele-specific assay. A 177-bp region containing this sequence variant was amplified from genomic DNA obtained from the other members of the myotonic goat colony and 11 additional normal goats. Digestion of this product amplified from normal goat DNA with *Mbo*II produces two bands of 105 and 72 bp, whereas digestion of DNA from all of the myotonic goats results in three bands (72, 53, and 52 bp), indicating the presence of the sequence variant in all members of the inbred colony (Fig. 2*D*). The absence of the 105-bp fragment further indicates that each myotonic goat is homozygous for the transversion. This sequence variant occurs within a 7-amino acid segment that is completely conserved in the carboxyl terminus of ClC-1 (all species), ClC-2, and ClC-0 (Fig. 2*C*), and is, therefore, a candidate mutation.

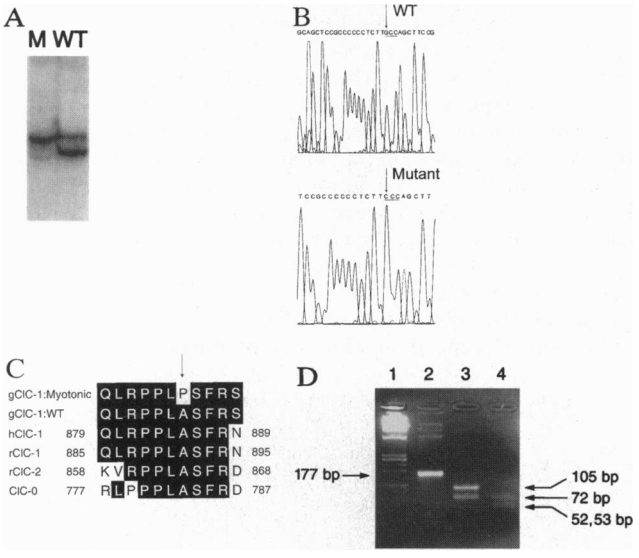

FIG. 2. Missense mutation in myotonic goat gClC-1. (*A*) Autoradiograph showing a single-strand conformational polymorphism in myotonic goat gClC-1 located within a cDNA segment encoding the carboxyl terminus. (*B*) Nucleotide sequence chromatograph showing the position of a G → C mutation in myotonic goat gClC-1. The mutation is indicated by an arrow labeled "Mutant." The sequence of WT gClC-1 in the same region is also shown. (*C*) Amino acid alignment of various ClC Cl^- channels in the region surrounding the myotonic goat mutation (indicated by arrow). Identical residues are shown by white-on-black type. (*D*) Allele-specific restriction endonuclease digestion of WT and myotonic goat PCR-amplified genomic DNA. Lanes: 1, molecular weight standard (ΦX174 *Hae*III digest); 2, undigested goat DNA; 3, *Mbo*II-digested WT goat DNA; 4, *Mbo*II-digested myotonic goat DNA. Sizes of each band in bp are indicated.

If this sequence variant is a mutation, then we expect the mutant allele to exhibit a functional abnormality sufficient to explain the decreased g_{Cl} observed in myotonic muscle. To ascertain the functional consequences of this substitution, we engineered the mutation (A885P) in hClC-1 and examined its functional properties in *Xenopus* oocytes by two-electrode voltage clamp recording. Fig. 3 shows representative traces of WT hClC-1 and the A885P mutant along with their respective current–voltage relationships. Peak current amplitudes were similar in both WT and A885P expressing oocytes, indicating similar expression levels of the two alleles. Upon hyperpolarization, the WT channel (Fig. 3*A*) deactivates to a non-zero steady-state level. With more depolarizing potentials, the steady-state current amplitude first increases and then decreases, producing the cross-over pattern that is characteristic of ClC-1 at negative potentials (13, 20). At potentials positive to −65 mV, the current is almost time-independent. The A885P mutant (Fig. 3*B*) also deactivates to a constant level upon hyperpolarization, but the "crossing over" of the steady-state current amplitude is less pronounced and occurs at potentials positive to −30 mV (versus −65 mV for WT). Upon depolarizing test potentials, there is a more pronounced time-dependent increase of the current amplitude with the A885P mutant than with WT.

Fig. 3 *C* and *D* show the current–voltage relationships for WT and A885P. The instantaneous current is inwardly rectifying for both WT and mutant and mean instantaneous current amplitudes are not different. Currents for both WT and A885P "cross over" at −30 mV, which is the reversal potential for Cl^- in these experiments. In contrast, the voltage dependence of the steady-state current for A885P is different from WT. Whereas with WT, instantaneous and steady-state current amplitudes are identical at potentials positive to the Cl^- equilibrium potential, A885P current amplitudes at the end of the test pulse are larger than the instantaneous values.

The results presented in Fig. 3 suggest a difference in the voltage dependence of gating between WT and the A885P mutant. Therefore, we evaluated the steady-state activation of the WT and mutant channels at voltages ranging from −145 mV to +35 mV by comparing their relative open probabilities (Fig. 4). In these experiments, the A885P mutant channel exhibits a dramatic and significant +47-mV shift in the midpoint of steady-state activation [WT, -64.1 ± 6.0 mV ($n = 4$); A885P, -17.2 ± 7.7 mV ($n = 6$); $p < 0.005$), but no change in slope factor (see Fig. 4). This shift results in a substantially decreased open probability of the mutant relative to WT within the physiological voltage range and is the likely cause of the decreased g_{Cl} in myotonic muscle. Therefore, the alanine to proline substitution identified in the myotonic goat ClC-1 carboxyl terminus is consistent with a mutation causing myotonia.

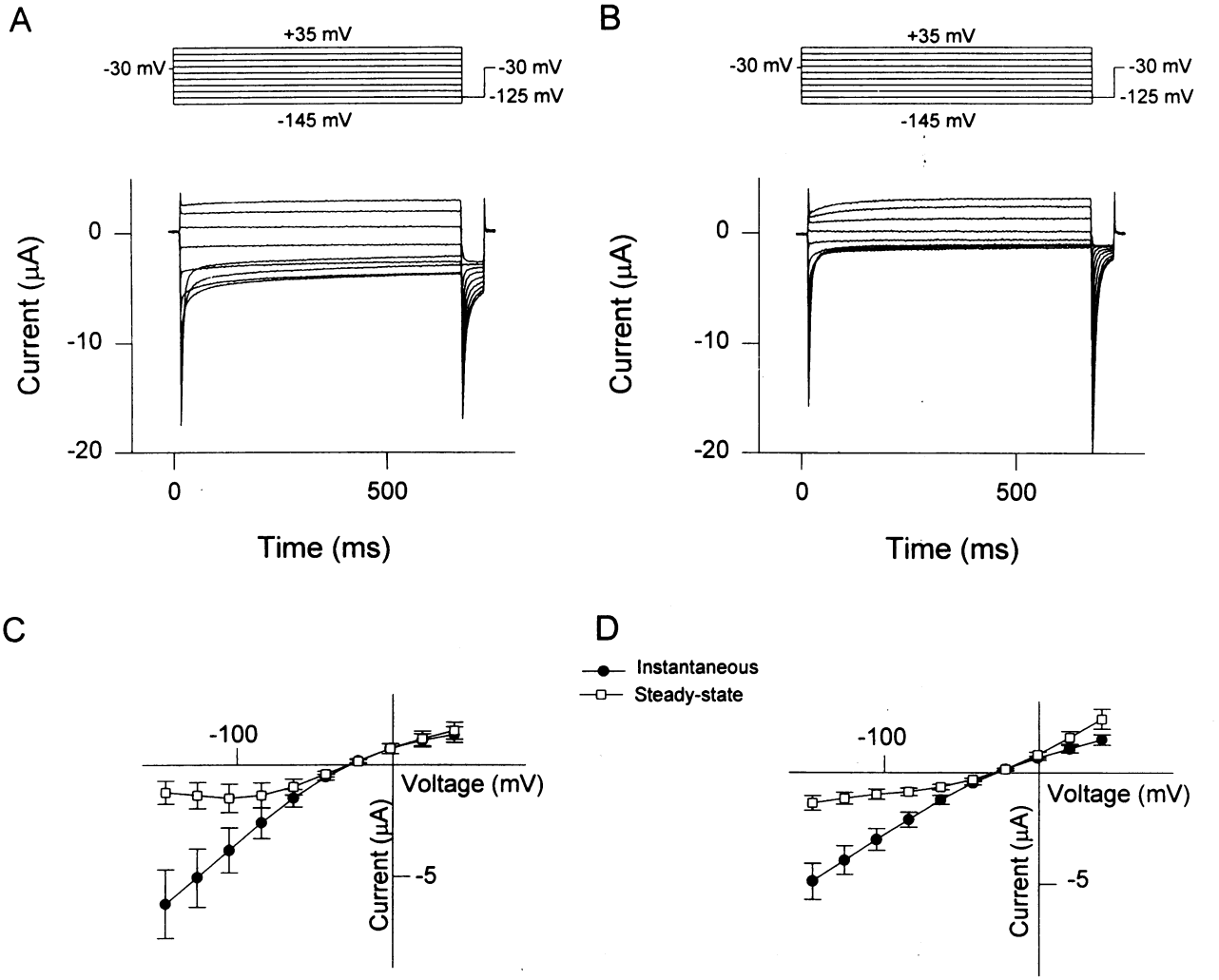

FIG. 3. Heterologous expression and current–voltage relations of WT and A885P hClC-1 channels. (*A* and *B*) Current responses to voltage steps from a holding potential of −30 mV in 20-mV steps from −145 mV to +35 mV are shown. Test pulses are followed by a voltage step to −125 mV. *Xenopus* oocytes were injected with WT hClC-1 (*A*) or A885P (*B*) RNA. (*C* and *D*) Voltage dependence of instantaneous (solid circles) and steady-state (open squares) current amplitudes in WT (*C*) and A885P (*D*) expressed in oocytes. Data are means ± SEM; $n = 8$ (WT) and $n = 10$ (A885P).

DISCUSSION

In this paper we have defined the molecular genetic basis of myotonia that occurs in the goat and demonstrate the functional consequences of the disease-producing mutation using a recombinant chloride channel. The mutation we described causes the substitution of a highly conserved alanine residue with proline within the predicted cytoplasmic carboxyl terminus region of the ClC-1 protein. This missense mutation occurs within a short sequence of 7 amino acids that is completely conserved in a group of closely related ClC channels including human and rat ClC-1 (10, 21), ClC-2 (22), and the *Torpedo* electroplax channel ClC-0 (23), although many subfamilies of ClC channels lack homology to ClC-1 within the carboxyl terminus (24–27). Other myotonia-producing mutations have been described in the human ClC-1 carboxyl terminus including two nonsense mutations (ref. 28 and unpublished observations).

In the absence of a full-length gClC-1 cDNA, the functional consequences of the myotonic goat ClC-1 mutation were studied in the well-characterized human homologue hClC-1 that was expressed in *Xenopus* oocytes. When compared with WT hClC-1, the mutant channel exhibits a dramatic shift in voltage dependence of activation along the voltage axis. Similar functional disturbances in hClC-1 have been described for mutations causing autosomal dominant human myotonia congenita (29). Four hClC-1 missense mutations (I290M, R317Q, P480L, and Q552R) occurring in diverse locations within the channel shift steady-state open probability toward more depolarized potentials when expressed in oocytes either alone or in combination with equal quantities of the WT channel. It would thus appear that a common biophysical phenotype can cause myotonia. This is reminiscent of the human disease paramyotonia congenita due to a variety of mutations in the skeletal muscle Na^+ channel that cause a similar pattern of functional disturbances in that channel (30). Mutations associated with dominant myotonia congenita exert a dominant-negative effect on the Cl^- channel most likely because of incorporation of dysfunctional subunits into a multimeric channel complex (12).

The physiological consequences of the shift in the voltage dependence of channel activation will be a decreased open probability of the chloride channel and a resultant decreased g_{Cl} in a voltage range around the electrical threshold for action potential generation in muscle. As discussed by Bryant (31), muscle fibers with diminished g_{Cl} require a smaller electrical stimulus to elicit an action potential (increased excitability) due to the absence of this conductance. Furthermore, during impulse propagation in the transverse tubules (t-tubule), a rise in extracellular potassium occurs that results in a small residual membrane depolarization after action potential termination

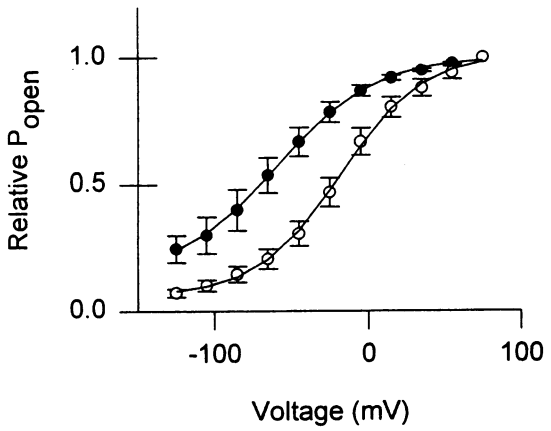

FIG. 4. Voltage dependence of activation for WT and A885P channels expressed in oocytes. Normalized instantaneous current amplitudes (relative P_{open}) for WT (solid circles) and A885P (open squares) were plotted versus the prepulse potentials. Solid lines represent fits of single Boltzmann distributions to the data. Fit parameters are as follows: $V_{0.5}$: WT, -64.1 ± 6.0 mV; A885P, -17.2 ± 7.7 mV. k_V: WT, -30.3 ± 1.2 mV; A885P, -24.9 ± 1.9 mV. The slope factors (k_V) are not statistically different between WT and A885P ($P > 0.06$, unpaired t test). To normalize the maximum relative P_{open}, the data points were scaled so that the fitted function had an asymptote of unity. Data are means ± SEM; $n = 4$ (WT) and $n = 6$ (A885P).

(7). Normally, this rise in extracellular potassium in the t-tubules has little effect on membrane potential due to the shunting effect of a high sarcolemmal g_{Cl}. By contrast in myotonic muscle, the rise in t-tubular potassium has a 10-fold greater effect on the membrane potential and results in a significant degree of depolarization after action potential termination ("after-depolarization"). If sufficient numbers of impulses are propagated rapidly in the t-tubule, then the after-depolarization can achieve threshold voltage and cause the spontaneous triggering of action potentials independent of neuromuscular transmission. These events will result in clinical myotonia.

With definition of the molecular defect responsible for the myotonic goat, our work provides a direct explanation for the sentinel observations made by Bryant and colleagues (5, 6) more than three decades ago and contributes to the union of cellular and molecular physiology in understanding myotonia.

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